The specification enclosed herewith is the duly verified English translation of the Korean Application No.: 10-2002-0003184

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[ABSTRACT]

This invention is about a novel Biomolecule Transducton Motif (BTM), Sim-2 peptide, which has a potential to transduce many biological response modifiers effectively into the cytoplasm or nucleus of prokaryotic or eukaryotic cells in vivo and in vitro, and the related technological methods using the Sim-2 BTM. The **Sim-2** BTM of the present invention, which includes amino acid sequence of SEQ. ID No.: 1, delivers biologically active functional regulatory molecules *in vivo* and *in vitro* into the cytosol, or nucleus of prokaryotic and eukaryotic cells, via numerous paths, for example, intramascular, intraperitoneal, intravein, oral, nasal, subcutaneous, intradermal, mucosal, and inhalation. This Sim-2 BTM can be used for Drug Delivery System, development of new recombinant protein vaccines or DNA/RNA vaccines, gene and protein therapy, production of pharmacologically or medicinally useful proteins or pharmacomedicinal drug therapy.

[Representative Figure]

Fig. 1a

[DESCRIPTION]

[Invention Title]

BIOMOLECULE TRANSDUCTION PEPTIDE SIM2-BTM AND BIOTECHNOLOGICAL PRODUCTS INCLUDING IT

[Brief Description of Figures]

SEQ ID NO. 1 is an amino acid sequence of SIM-2-BTM.

SEQ ID NO. 2 is a nucleotide sequence to which Gal4 binding protein binds.

SEQ ID NO. 3 is a nucleotide sequence of 5' primer to prepare pSIM2-β-gal.

SEQ ID NO. 4 is a nucleotide sequence of 3' primer to prepare pSIM2-β-gal.

Fig.1a illustrates the construct of expression vector pSim-2-β-gal.

Fig.1b shows agarose gel images after treating the vector of Fig.1A with restriction enzymes.

Fig.2a shows coomasie blue staining of the purified fusion proteins expressed from pSim-2-β-gal vector.

Fig.3 shows the result of β-gal enzyme activity analysis proving that the fusion protein of Sim-2-β-gal has been effectively transduced into cells.

Fig.4a illustrates the construct of expression vector of pTat-β-gal.

Fig. 4b shows the result of β-gal enzyme acitivity analysis proving that the fusion protein of SIM2 and β-gal has been more effectively transduced in to cells than the fusion protein of the existing Protein Transduction Domain(PTD), Tat and β-gal.

Fig. 5 illustrates the effect of transduction into cells in vivo by BTM SIM-2.

Fig.6a illustrates the recombinant expression vector of pSim-2-β-gal-B7.1.

Fig.6b shows the results of FACS analysis, representing that the desired molecule of β-gal is specifically transduced into T cells.

Fig.7 illustrates the construct of recombinant expression vector of pSim-2-Gal4.

Fig.8 illustrates the construct of recombinant expression vector of pCD8-ζ-3XGBS (Gal4 binding sequence).

Fig. 9 illustrates the transduction of an expression vector CD8-ζ DNA into cells by SIM2 biomolecule transduction peptide.

Fig.10a illustrates the construct of expression vector of pSim-2-Gal4-B7.1.

Fig.10b illustrates the construct of expression vector of pLCD8-ζ-3XGBS.

Fig.10c represents T cell specific transduction of expression vector pCD8-ζ-3XGBS using Sim-2 BTM.

Fig.11 illustrates the transduction of TMC into cells by SIM2 biomolecule transduction peptide.

[Disclosure]

[Objective of Invention]

[Technical Field and Background Art]

This invention relates to Sim-2 Biomolecule Transduction Motif (BTM), a novel intracellular biomolecule transduction peptide, which delivers biologically active, functional or/and regulatory molecules *in vivo* and *in vitro* into the cytosol or nucleus of prokaryotic and eukaryotic cells, and a method for transducing the same into cytosol or nucleus of cell.

Generally, living cells are not permeable to macromolecules such as proteins or nucleic acids. The fact that only small-size molecules can permeate through the membrane of living cells at very low rates has restricted the researches to develop drugs to cure, prevent or diagnose diseases using macromolecules including, for example, proteins and nucleic acids. On the other hand, because most of the substances manufactured to cure,

prevent or diagnose certain diseases have to be delivered into the cytosol with effective amounts, there have been several methods developed delivering those substances from the cell surface of the target cell into the cell. Methods used to deliver macromolecules into cells include electroporation, cytosol fusion using liposomes, highly concentrated projection technique of a projectile coated with DNA on surface, calcium-phosphorous-DNA precipitation method, DEAE-dextran transfection, infection with modified viral nucleic acids, and direct microinjection into a single cell, etc. Furthermore, these methods can deliver macromolecules to only a few of the target cells, and its efficiency is not sufficient enough to be applied clinically. Also, most of these methods cause side effects on the other cells.

In this regard, the demand for the development of a novel method of delivering biologically active macromolecules into the target cells effectively *in vivo* and *in vitro* is increasing (L.A. Sternson, Ann. N.Y. Acad. Sci., 57, 19-21(1987)). Chemical addition of lipid peptide (P. Hoffmann et al. Immunobiol., 177, 158-170(1988)) or use of base polymers such as polylysine or polyarginine (W-C. Chen et al., Proc. Natl. Acad Sci., USA. 75, 1872-1876(1978)) were provided. In addition, it was reported that folate of a transporter was transferred into a cell in the form of folate-conjugate. But it has not been confirmed yet that the folate was transduced into the cytosol. Also, Pseudomonas Exotoxin is known as a transporter (T. I. Prior et al., Cell. 64, 1017-1023(1991)). However, the effects of biologically active macromolecules delivered into the cytosol and their general application have not been clearly verified yet. Therefore, an effective method of transducing biologically active macromolecules into cytosol and nucleus of living cells is highly demanded.

Several PTDs (Protein Transduction Domain) have been reported as a result of this demand. Among them, Tat protein, which is a Human Immunodeficiency Virus-1

(HIV-1) viral protein, has been mostly well studied. The Tat protein was known to operate more efficiently when containing amino acids 47 to 57 (YGRKKRRQRRR), where positive charged amino acids are concentrated, than containing full-length 86 amino acid protein (Fawell S. et al. Proc. Natl. Acad. Sci. USA 91, 664-668(1994)). Other examples of PTDs are amino acids 267 to 300 of Herpes Simplex Virus type 1 protein (HSV-1) (Elliott G. et al. Cell, 88, 223-233(1997)), amino acids 339 to 355 of Antennapedia (ANTP) protein of Drosophila (Schwarze S. R. et al. Trends Pharmacol Sci. 21, 45-48(2000)), and artificial combination of positively charged amino acids. Regarding the PTDs mentioned above, we, inventors, found that they contained lysine and arginine abundantly, wherein the arginine was considered to play a great role in the transduction of biomolecule into cells. And it was supported by the published document that disclosed transduction activities of artificial peptides consisting of positively charged amino acids. (Laus R. et al. Nature Biotechnol. 18, 1269-1272(2000)).

However, experiments with artificially combined amino acids with 12 arginines and 12 lysines suggested that the hypothesis might be wrong. Therefore, PTDs would be found frequently in transcription factors. And the PTDs may use channels similar to translocons that transmit proteins into organelles.

Therefore, we found that human SIM-2 can transduce proteins after searching candidate genes having cluster of basic amino acid in Genbank and studying their transcription factors, based upon the fact that many motif in transcriptional factor such as arginine and lysine in various signal peptides which make proteins transducing into nucleous or mitochondria.

Amino acid sequence 558 to 566 of Sim-2, a human transcription factor, was found to have unexpectedly significant transduction ability and was named Sim-2 BTM (Biomolecule Transduction Motif).

Thus, we completed the present invention with the findings that amino acids 558 to 566 of Sim-2 have significantly excellent features as an intracellular biomolecule transduction peptide, thereby any target proteins, nucleic acids, lipids, carbohydrates and chemical compounds can be efficiently delivered into cytosol and nucleus *in vivo* and *in vitro*.

[Technical Problem]

The object of this invention is to provide a novel biomolecule transduction motif Sim-2-BTM (SEQ.ID No.:1), which effectively transduce biologically active, functional regulatory macromolecules *in vivo* and *in vitro* via numerous administration routes including intramascular, intraperitoneal, intravein, oral, nasal, subcutaneous, intradermal, mucosal, and inhalation routes and to provide the recombinant expression vector including it.

Another object of this invention is to provide recombinant expression vector comprising Sim-2-BTM and to provide transformed cells using the vector.

Another object of this invention is to provide the fusion protein of biomolecule transduction motif and a desired protein by transforming a suitable host cell with the recombinant expression vector.

Another object of this invention is to provide a method of transducing biologically active functional modulatory macromolecule *in vivo* and *in vitro* into the cytosol or nucleus of prokaryotes or eukaryotes using Sim-2-BTM.

Another object of this invention is to use the Sim-2 BTM in gene therapies and in disease treatment using proteins.

[Mode for Invention]

In order to achieve these objects, this invention provides a peptide or its active fragment comprising amino acid sequence (SEQ. ID No.:1) to transduce biologically active functional modulatory macromolecules *in vivo* and *in vitro* into the cytosol or nucleus of prokaryotes or eukaryotes.

Furthermore, this invention provides a peptide or its active fragment comprising amino acid sequence (SEQ. ID No.:1) to transduce biologically active functional modulatory macromolecules *in vivo* and *in vitro* into the cytosol or nucleus of prokaryotes or eukaryotes, wherein a part of the amino acids is deleted or substituted.

Furthermore, this invention provides DNA encoding intracellular biomolecule transduction peptide, recombinant DNA expression vector comprising DNAs encoding target protein, and transformed E. coli of DH5a Sim-2 (KCCM 10346) using the vector.

Furthermore, this invention provides a method of transducing a complex of the peptide (or the peptide fused with a target protein) and biologically active functional regulatory molecule (for example, chemical drug or chemical prodrug) via numerous administration routes includinding intramuscular, intraperitoneal, intravenous, oral, nasal, subcutaneous, intradermal, mucosal and inhalation routes, *in vivo* and *in vitro* into the cytosol or nucleus of prokaryotes or eukaryotes. The following describes this invention in detail.

Below are more detailed descriptions.

In this specification and claims, "biologically active functional regulatory molecule" means the molecule, which regulates any physiological phenomenon, including, for example, DNA, RNA, protein, lipids, carbohydrates, and chemical compounds.

In addition, "an active fragment" is defined as some parts of amino acids of SEQ. ID No.:1 or its modified one in which a part of the amino acids is deleted or substituted, wherein the fragment maintains intracellular biomolecule transduction activites.

In addition, "macromolecule" is defined as to include proteins, lipids, nucleic acids, carbohydrates and chemical compounds.

In addition, a biomolecule transduction peptide of "Sim-2-BTM derivative" is defined as a peptide or its active fragment, which maintains transduction activites of Sim-2-BTM, wherein a part of the amino acids is deleted or substituted.

In addition, a "fusion protein" is defined as a protein fused with biomolecule transduction peptide of Sim-2-BTM directly through chemical, physical, covalent or non-covalent bond, or indirectly through any mediator.

In addition, a "chemical compound" is defined as a chemical substance, which regulates cell's function, such as anticancer drug, immunological disease drug, antiviral drug and growth, development or differentiation factor of an animal.

In this invention, a biomolecule transduction peptide is defined as a peptide corresponding to amino acids from 558th to 566th from N-terminus of human transcription inhibitor Sim-2 (M.J Alkema et al., Genes Dev. 12), 226-240(1997)), wherein arginines and alanines are considered as to interact with biomolecule transducing channel receptors on the cell surface.

In addition, this invention relates to a peptide corresponding to the amino acid sequence of SEQ. ID No.:1, wherein a portion of amino acid including arginine, lysine and alanine is substituted with functionally and structurally similar amino acids, for example, valine, etc.

This invention also provides an expression vector comprising: i) DNA/RNA encoding a biomolecule transduction peptide corresponding to amino acid sequence of

SEQ. ID No.:1, or DNA/RNA encoding a biomolecule transduction peptide corresponding to modified amino acid sequence of SEQ. ID No.:1 in which a part of the amino acids is deleted or substituted, or DNA/RNA encoding an active fragment of the peptide; and DNA/RNA encoding a desired protein and/or biologically active functional regulatory molecules to be introduced into cells. This recombinant expression vector preferably includes tag sequence(s), such as a series of Histidine, Hemaglutinin, Myc, or Maltose binding protein codon, and so forth, in order to make the purification easier. Furthermore, a cleavage site recognized by a protease specifically present in a certain intracellular organelle to remove unwanted part of the fusion protein or to separate BTM and the cargo molecule, expression regulatory sequence and a marker to monitor the transduction or a reporter gene can be inserted to the expression vector, wherein the expression regulatory sequence consists of regulatory domain comprising a promoter or enhancer that is specific to cells, tissues or organs to which the desired DNA/RNA is transduced.

In one embodiment, recombinant expression vector including intracellular biomolecule transduction peptide of pSim-2-β-gal comprises DNAs encoding a peptide corresponding to amino acid sequence of SEQ. ID No.:1, 6 (six) succesive histidine codons to purify the desired proteins expressed in host cells, Asp-Asp-Asp-Asp-Lys sequence to be cleaved with enterokinase and DNAs encoding a marker of β-galactosidase for the detection of the desired protein in cells.

The pSim-2-β-gal vector of this invention can easily be obtained with ordinary PCR (polymerase chain reaction) methods using pIND/lacZ vector (can be obtained from Invitrogen Inc.) as a template. Also, in this invention, biomolecule transducing recombinant expression vector is manufactured by cutting out β-galactosidase gene with suitable restriction enzyme and replacing it with the desired protein coding DNAs. The desired protein comprises biologically active functional regulatory protein or its fused one,

which is chemically or physically bound to ecto domain of ligand that binds specifically to a receptor of cell, tissue or organ to which the desired protein is introduced, in order to transduce it to specific cell or tissue or organ. This ligand or receptor includes protein, lipid, carbohydrate, chemical compound or its complex, but is not limited thereto. Biomolucule of this invention incldes DNA/RNA, carbohydrate, lipid, protein or chemical compounds through chemical, physical, covalent or no-covalent bond, but not limited to this range.

In one embodiment of the present invention, the desired protein is isolated and purified using the recombinant expression vector. Specifically, after transforming a suitable host cell, such as E. coli, with the recombinant expression vector of this invention, the desired protein is isolated using interaction between polyhistidine and Ni^{2+}-NTA.

Furthermore, in another embodiment, a method for transducing functional regulatory molecules are transduced more effectively into cytosol, organelle or nucleus by culturing the recombinant expression vector of the present invention together with biologically active functional regulatory molecules.

In another embodiment, a method of transducing biomolecules is provided as follows: i) providing recombinant expression vector comprising DNAs encoding DNA/RNA binding protein that binds selectively to DBS/RBS (DNA/RNA binding sequence) of the desired DNA/RNA; ii) obtaining protein-DNA/RNA complex by combining the desired DNA/RNA sequence, which contains target DNA/RNA sequence that binds to the DNA/RNA binding protein; and iii) mixed culturing the protein-DNA/RNA complex with cell culture medium in order to transduce the desired DNA/RNA into the cells.

In another embodiment, a method of tranasducing biomolecule is provided, which comprises: i) obtaining a complex by reacting fusion proteins, which are activated by binding inducer, with the desired chemical compound, wherein the fusion protein is a fused

one between the biomolecule transduction peptide of Sim-2BTM or its derivatives and the desired protein; and ii) mixed culturing the obtained complex together with cell culture medium in order to transduce the desired chemical compounds into the cells. The binding inducers introduced above include binding reagents, for example, BMOE (Pierce Cat. No 2323), DSP (Pierce Cat. No 22585), that bind the biomolecule transduction peptide or the fusion protein between the transduction peptide and the desired protein to DNA/RNA, carbohydrate, lipid, protein or chemical compounds through chemical, physical, covalent or no-covalent bond, directly or indirectly.

In addition, in this invention, when transducing the desired DNA/RNA, or chemical compound into cells, they are combined with the fusion protein between the biomolecule transducing peptide and the desired protein, wherein the desired protein can form a transduction complex by binding the ecto domain of a ligand, which selectively interacts with a receptor that is expressed in specific cells, organs and tissues, or mAb or its mutant.

Since the biomolecule transducing peptide of the present invention is very small, biological intervention to the active molecule is significantly minimized.

Below are more detailed descriptions according to practical examples. Below practical examples are there to show examples, and this invention is not limited to this range.

EXAMPLE 1: Preparation of recombinant expression vector including Sim-2-BTM of biomolecule transduction peptide

Nucleic acid sequence encoding peptide corresponding to amino acids from 558th of Alanin to 566th of Arginin from N-terminus mouse transcription factor of **Sim-2**

(GeneBank Code: NM_0050969, gi: 7108363) was combined with nucleic acid sequence encoding reporter protein of β-galactosidase. In order to do this, firstly, a primer of SEQ.ID No.:2 containing nucleic acid sequence encoding peptide corresponding to amino acids from 558th of Alanin to 566th of Arginin from N-terminus of **Sim-2** and *BamHI* site for cloning, and a primer of SEQ.ID No.:3 containing nucleic acid sequence of 3' terminus of β-galactosidase and restriction enzyme of BglII site for cloning were synthesized. Then, PCR was carried out using pIND/lacZ vector (Invitrogen corp.), as a template, with pfu turbo DNA polymerase (Stratagene, cat.# 600252-51).

After purifying the PCR product with PCR purification kit (Quiaquick) (QIAGEN, cat.# 28104), the result was digested with BglII and BamHI restriction enzymes for 48 hours. And then, it was subject to electrophoresis on 1% agarose gel followed by staining with ethydium bromide (see Fig.1b). In Fig.1b, the first column represents the present p **Sim-2-**β-gal, and the second column represents standard sized DNA fragments.

After digested the PCR product with restriction enzyme BglII, the products were cloned to pTrcHis B (Invitrogen, Cat.No. V360-20B), which was purified with gel extraction, at Bg1II recognition site, thereby recombinant expression vector was generated and named p**Sim-2-**β-gal. Fig.1a illustrates the construct of the expression vector of p**Sim-2-**β-gal.

EXAMPLE 2: Preparation of E.coli transformant, and expression and isolation of fusion protein therefrom

DH5 α (ATCC No. 53863) was transformed using p**Sim-2-**β-gal of the Example

1 by heat shock transformation. 2 ml of the transformants were transferred to 100 ㎖ of LB medium, and pre-incubated for 12 hrs at 37℃ with agitation. Then, the pre-incubated transformants were inoculated to 1000 ㎖ of LB medium and were incubated for 4 hrs at 37℃. Following the incubation, 1 mM of IPTG (Isopropyl β-D-thiogalactosipyranoside, GibcoBRL cat.# 15529-019) was added in order to induce the expression of lac operon, and then incubated again for 8 hrs to elicit expression of the fusion protein.

Next, the cell culture solution was centrifuged at 6,000 rpm for 20 min at 4℃. After removing the supernatant, resultant pellet was dissolved in 10 ml of buffer solution 1 (50 mM of NaH_2PO_4, 300 mM of NaCl, 10 mM of Imidazole, at pH 8.0) including 1 ㎎/㎖ of lysozyme (Sigma, cat.# L-7651), and was placed on ice for 30 min. Subsequently, ultrasonification (Heat systems, ultrasonic processor XL, with 300 w) for 10 sec and freezing for 10 sec were applied to the solution, repeatedly, until the cumulative time of ultrasonification is 3 min. The eluted solution was centrifuged again at 12,000 rpm for 20 min at 4℃ so as to remove the debris of E. coli and isolate pure eluted solution.

Then, 2.5 ㎖ of 50% Ni^{2+}-NTA agarose slurry (Qiagen, cat# 30230) was added to the isolated solution, and the mixture was stirred at 200 rpm for 1 hr at 4℃ in order to bind fusion protein and Ni^{2+}-NTA agarose. The obtained mixture was spilt into a column (0.8×4 cm) for chromatography (BioRad, cat.# 731-1550).

And the mixture was washed twice with 4 ㎖ of buffer solution 2 (50 mM of NaH_2PO_4, 300 mM of NaCl, 20 mM of Imidazole, at pH 8.0). Thereafter, the mixture solution was treated four times with 0.5 ㎖ of buffer solution 3 (50 mM of NaH_2PO_4, 300 mM of NaCl, 250 mM of Imidazole, at pH 8.0) to obtain fusion protein fractions. The isolated and purified **Sim-2-** β-gal fusion protein was subject to SDS-PAGE followed by coomassie blue staining (see Fig.2). In Fig.2, the first column corresponds to protein marker for use as a molecular weight standard, and the second column represents fusion

protein of **Sim-2-β-gal.**

In order to examine β-galactosidase activity of purely isolated and purified fusion protein, absorbace was measured in 405nm with ONPG(o-nitrophenyl-β-galactopyranoside) as substrate and we observed enzyme activity of the fusion protein (Fig.2b). As shown in Fig.2b, the purified fusion protein showed high β-gal activity, lysate(-) of E. coli without expression vection showed little enzyme activity.

EXAMPLE 3: Transduction of fusion protein across cell membrane

5×10^5 of Jurkat cell (obtained from ATCC No. TIB-152) was diluted with the fusion protein obtained in the Example 2 to 1ug/ml in PBS, and 1 ml of the resultant was poured in 35mm Petri-dish and reacted for 30 minutes in 37 ℃ or 4℃.

After termination of the reaction, cells were harvested and reacted with 100㎕ of elution buffer solution (0.2% Triton X-100, 150 mM of NaCl, 10 mM of Tris-HCl, 400 μM of EDTA, 1 mM of Na_3VO_4, 10 mM of NaF, 1mM PMSF, 10 μg of aprotinin, 10 μg of leupeptin) for 30 minutes in 4℃ and then cell elute was obtained by centrifugation for 15 minutes at 14,000rpm.

Sample solution for measuring activity (3 μl of $100\times Mg^{2+}$ solution, ONPG) 66㎕ and 0.1M of sodium phosphate, and then was subjected to reaction for 30 min at 37℃ followed by the addition of 1 M of Na_2CO_3 500㎕ thereto. Absorbance of solution (at 405 nm) was measured three times with microplate reader (Molecular devices) and its mean values and stand deviations were represented in Fig.3. As the results, it was observed that **Sim-2-β-gal** fusion protein was very efficiently transduced across cell membrane both at 37℃ and at 4℃, which clarified that the protein transduction using the Sim-2 of the present invention did not merely result from receptor-mediated endocytosis or

phagocytosis.

EXAMPLE 4: Comparison of transduction efficiencies across cell membrane between PTD Tat and Sim-2

A pTat-β-gal DNA construct was designed in order to compare the protein transduction efficiencies across cell membrane between the conventional PTD or Tat and **Sim-2** of the present invention. Fig. 5a illustrates the construct. After isolation and purification of Tat-β-gal fusion protein according to the Example 2, transduction efficiencies of Tat-β-gal and **Sim-2**-β-gal into cells were compared. As disclosed in Fig. 5b, **Sim-2** was transduced into the cells as about 50 times comparing with Tat.

EXAMPLE 5: *In vivo* transduction of a desired protein using Sim-2

Transduction efficiency of a desired protein using **Sim-2** across cell membrane *in vivo* was detected in this example. In order to detect the efficiency, the isolated and purified 750 ug of **Sim-2**-β-gal fusion protein of the Example 3 was mixed with PBS, and 0.5mg/ml of the mixture was administered by IP (intra peritoneal) injection into C57BL/6 mice. As shown in Fig. 6, high activity of β-gal enzyme in liver and spleen was detected, which supported that the target protein was efficiently transferred into organs *in vivo* using **Sim-2**-BTM.

EXAMPLE 6: Cell specific transduction of fusion protein between biomolecule transduction peptide and a desired protein

In order for the desired protein of **Sim-2-β-gal** according to the Example 2 to be introduce to a specific cell selectively, a ligand or a receptor that exists specifically in cell, tissue or organ to which the protein is transduced, was employed in this example. For example, in order to transduce the desired protein to T cell, an expression vector of p**Sim-2-β-gal-B7.1** was designed by inserting amino acid sequence of MMP (MatrixMetalloprotease) cleavage site, which was present on extracellular matrix and was attached to cell membrane, into **Sim-2-β-gal** at 3' region, and then by cloning B7.1 of ligand of T cell specific CD28 receptor, next to the 3' region. Fig.6a represents the construct of the expression vector of p**Sim-2-β-gal-B7.1**. After transforming DH5a with the p**Sim-2-β-gal-B7.1** expression vector, fusion protein of **Sim-2-β-gal-B7.1** was isolated and purified from the cell culture. After 4 hrs from the I.P. injection of the fusion protein to a mouse, T cells were collected from blood to detect activity of β-gal. As disclosed in Fig.6B, relatively high β-gal activity was observed in T cells, while little β-gal activity was observed in B cells.

EXAMPLE 7: Intracellular transduction of DNA (CD8-ζ) using Sim-2

(STEP 1) Preparation of expression vector including fusion genes of Sim-2 and Gal4

The p**Sim-2-β-gal** vector of the Example 1 was treated with restriction enzymes, *Xba*I and *Bgl*II, to remove β-galactosidase gene therefrom. Then, Gal4 gene (obtained from Professor Choi, Kang Yoel, Dept. of Biotechnology, Yonsei University, Korea) was inserted to prepare p**Sim-2-Gal4** plasmid, wherein the gene was modified to have

restriction recognition sites of XbaI and BglII in 5' terminus and 3' terminus respectively. Fig.7 illustrates the construct of the expression vector of p**Sim-2**-Gal4.

(STEP 2) Preparation of expression vector including DNA sequences to which CD8- ζ and Gal4 bind

In order for the binding of Gal4 to be carried out more efficiently, pCD8- ζ - 3XGBS fusion DNA construct was designed by cloning 3 (three) successive GBS sequence(Gal4 binding sequence) at the BamHI restriction enzyme recognition site of pcDNA3 expression vector(obtained from Invitrogen), into which CD8- ζ was inserted at restriction enzyme recognition sites for *Xba*I and *Bam*HI. Specifically, nucleic acid sequence corresponding to GBS was synthesized using a primer and hybridized, and then was cloned to pCD8- ζ at BglII recognition site of 3' terminus after phosphorylation of 5' overhanging by kinase. Fig.8 illustrates the structure of expression vector of pCD8- ζ - 5XGBS. The nucleic acid sequence of GBS was designated as SEQ ID No.2.

(STEP 3) Confirmation of the transduction of CD8- ζ DNA using **Sim-2**

Gal4s of **Sim-2**-Gal4 fusion proteins were respectively bound to each of the three GBSs of pCD8- ζ -3XGBS. The expressed and purified **Sim-2**-Gal4 fusion protein in the Example 2 was bound to pCD8- ζ -3XGBS DNA prepared in the step 2 using the expression vector of p**Sim-2**-Gal4 manufactured in the step 1 at room temperature.

After mixing the fusion complex with PBS, the mixture was introduced to 10^7 of Jurkat cells, and then was incubated for 48 hrs at 37℃ in order to induce over- expression of CD8- ζ fusion proteins elicited by DNA constructs delivered into the cells. In order to

determine the over-expression of CD8- ζ fusion protein on the cell surface, FACS (Fluorescence-Activated Cell Sorter) analysis was carried out using OKT8 (ATCC No. CRL-8014) of a monoclonal antibody to CD8 (Current Protocol for Immunology). Fig.9 discloses the results. As shown in Fig.9, CD8- ζ fused with **Sim-2** biomolecule transduction peptide was over-expressed, and it suggests that the fusion protein of SIM2 and CD8- ζ was trasduced into the Jurkat cells as almost 100%. As (-)negative controls, the expressions of CD8- ζ chimeric molecules in Jurkat cells, which include **Sim-2**-Gal4 fusion proteins not having pCD8- ζ -3XGBS, were analyzed using FACS.

EXAMPLE 8: T cell specific transduction of pCD8- ζ DNA

(STEP 1) Preparation of an expression vector including genes of **Sim-2**, Gal4 and B7.1

T cell specific transduction of pCD8- ζ DNA using **Sim-2** was examined in this example. T cell specific MMP cleavage site and B7.1 were cloned to p**Sim-2**-Gal4 of the Example 7 at 3' region, according to the Example 4. Thereby, p**Sim-2**-Gal4-B7.1 DNA construct was generated . Fig. 10a illustrates the construct.

(STEP 2) Preparation of expression vector comprising T cell specific promoter of Lck, pCD8- ζ and 3 (three) GBSs

The promoter of pCD8-3XGBS, CMV promoter, which was designed in step 2 of the Example 7, was replaced by a T cell specific lck promoter at HindIII recognition site using conventional molecular cloning method. Fig.10b illustrates the construct of expression vector of pLCD8- ζ -3XGBS.

(STEP 3) T cell specific expression of pLCD8- ζ -3XGBS DNA using **Sim-2**

Using the expression vector pSim-2-Gal4-B7.1 DNA constructed in the step 1, the **Sim-2**-Gal4-B7.1 fusion protein, which was purified and expressed as disclosed in the Example 2, and the expression vector of pLCD8- ζ -3XGBS DNA, which was prepared in the step 2 were bound so that Gal4 of Sim2-Gal4-B7-1 and 3(three) of Gal4 binding sites in pLCD8- ζ -3XGBS were bound each other.

After the mixture of complex and PBS was inoculated in 10^7 Jurkat T cell and B cell, the resultant was cultured for 48 hours in 37℃, and then an over-expression of CD8- ζ fusion complex was induced by the DNA construct which was transduced into cells. Then, the expression level of surface chimeric molecule of CD8- ζ was examined with FACS (Fluorescence-Activated Cell Sorter) using anti-CD8 mAb of OKT8 (obtained from ATCC No CRL-8014) (Current Protocol for Immunology) (see Fig.10c). As shown in Fig. 10c, the CD8- ζ protein was over-expressed in only Jurkat T cell, which the CD8- ζ protein was transduced into cells by SIM2 biomolecule transduction peptide. (-) refers negative controls. After the fusion protein of pLCD8- ζ -3XGBS and SIM2-Gal4-B7.1 was reacted with B cells, the expression of CD8- ζ chimeric molecule was examined with FACS.

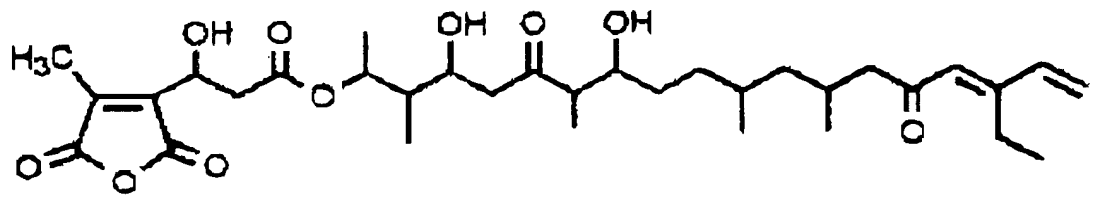
EXAMPLE 11: Intracellular transduction of fusion complex between Sim-2 and TMC

In order to prepare a fusion complex between SIM2- β -gal fusion protein, which was isolated and purified in Example 2, and TMC (Tautomycetin), which is an inducible

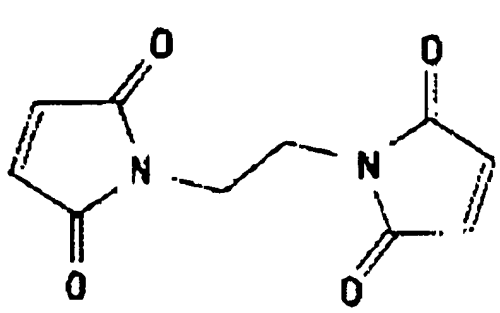
material of apoptosis in formula 1 below, SIM2-β-gal fusion protein was bound to binding inducible material BMOE (Pierce cat. #22323) of formula 2 below by activation procedure, and mixed with TMC, then a fusion between the activated constructs by reaction for 30 minutes in 37℃.

The resultant was mixed with culture of Jurkat cells and reacted for 1 hour so that TMC was transduced into cells. After 1 hour, death of cells was observed with Annexin V-P.I. staining (I. Schmid et. Al., Cytometry 13:204-208 (1992)). As a result, TMC, which was bound to SIM-2 and transduced into cells, induced apoptosis of cells and TMC, which was not bound to SIM-2, did not induce apoptosis of cells within the above time.

[Formula 1]



[Formula 2]



[Advantageous Effects]

As disclosed above, **Sim-2** BTM Sim-2 of the present invention, which includes amino acid sequence of SEQ. ID No.: 1, delivers biologically active functional regulatory molecules *in vivo* and *in vitro* into the cytosol, or nucleus of prokaryotic and eukaryotic cells, via numerous paths, for example, intramascular, intraperitoneal, intravein, oral, nasal, subcutaneous, intradermal, mucosal, and inhalation. Thus, this invention can be used to provide recombinant vaccines, DNA/RNA vaccines, and functional genes. Furthermore, this invention can be used to provide novel methods for treating disease using protein, genes, carbohydrates, lipids and chemical compounds pharmacomedicinal drug therapy.

[CLAIMS]

[Claim 1]

A peptide or its active fragment comprising amino acid sequence of SEQ ID NO.: 1

[Claim 2]

A peptide or its active fragment comprising amino acid sequence substituted with structurally and/or functionally similar amino acid, wherein the amino acid consist of one to three amino acids of SEQ ID NO.: 1.

[Claim 3]

The peptide or its active fragment of Claim 2, wherein the amino acid consist of at least one of arginine, lysine and alanine.

[Claim 4]

A peptide or its active fragment comprising amino acid sequence, wherein the amino acid can be included or deleted to maintain stability of entire structure and function in SEQ ID NO.: 1.

[Claim 5]

The peptide or its active fragment of any one of Claim 1 to 4, wherein a desired protein to be transduced into the cytoplasm or nucleus of prokaryotic or eukaryotic cells is fused or bound by chemic or physical method.

[Claim 6]

The peptide or its active fragment of Claim 5, wherein the desired protein is one or more homologous or heterologous protein which relates to biological activity regulation in vivo or in vitro.

[Claim 7]

The peptide or its active fragment of Claim 5 and 6, wherein the peptide or its active fragment bound to the desired protein is transduced into the cytoplasm or nucleus of prokaryotic or eukaryotic cells through routes such as intramuscular, intraperitoneal, intravein, oral, nasal, subcutaneous, intradermal, mucosal and inhale.

[Claim 8]

A recombinant expression vector to which the DNA encoding the peptide or its active fragment of any one of Claim 1 to 7, the DNA encoding the desired protein and expression regulation sequence to be transduced into cells are bound operatively.

[Claim 9]

The recombinant expression vector of Claim 8, wherein the desired protein is one or more homologous or heterologous protein which relates to biological activity regulation of cells in vivo or in vitro.

[Claim 10]

A recombinant expression vector for recombinant vaccine, wherein the DNA encoding the peptide or its active fragment of any one of Claim 1 to 7, the DNA encoding the desired protein to be tranduced into cells, the DNA encoding one or more ubiquitine, and expression regulation sequence are bound, wherein the desired protein is a fused virus-

specific protein or tumor-specific protein, wherein the vector change MHC class II mediated pathway to MHC class I mediated pathway in antigen processing in vivo to induce CTL(cytotoxic leukocyte).

[Claim 11]

The recombinant expression vector of Claim 10, wherein virus-specific protein is a viral protein from animal viruses comprising HIV, HBV, HCV, Influenza, and tumor-specific protein is a protein expressed specifically in cells of liver cancer or stomach cancer.

[Claim 12]

The recombinant expression vector of Claim 10, wherein the desired protein is one or more homologous or heterologous virus-specific protein or tumor-specific protein.

[Claim 13]

The recombinant expression vector of Claim 10 to 12, wherein the peptide or its active fragment bound to the desired protein is transduced into the cytoplasm or nucleus of prokaryotic or eukaryotic cells through routes comprising intramuscular, intraperitoneal, intravein, oral, nasal, subcutaneous, intradermal, mucosal, inhale.

[Claim 14]

The recombinant expression vector, wherein the vector has the DNA encoding the peptide or its active fragment of any one of Claim 1 to 7, the DNA encoding the desired protein to be transduced into cells, the amino acid sequence which is digested by protease (for example, MMP(MatrixMetalloProtease) on cell surface and the DNA encoding ligand or acceptor specifically existing in cells, tissue or organ into which the desired protein is

transduced orderly and is bound to expression regulation sequence operatively so that the desired protein of Claim 5 to 13 is transduced selectively into cells, tissue or organ.

[Claim 15]

The recombinant expression vector, wherein the vector has the DNA encoding the peptide or its active fragment of any one of Claim 1 to 7, the DNA encoding the desired protein to be transduced into cells, the amino acid sequence which is digested by protease (for example, MMP) on cell surface and the DNA encoding monoclonal antibody bound to ligand or acceptor specifically existing in cells, tissue or organ into which the desired protein is transduced orderly so that the desired protein of Claim 5 to 13 is transduced selectively into cells, tissue or organ.

[Claim 16]

A complex prepared by chemical covalent or noncovalent bond or physical method between the fusion protein of the peptide or its active fragment of any one of Claim 1 to 7, the desired protein to be transduced into cells, the amino acid sequence which is digested by protease (for example, MMP) on cell surface and the monoclonal antibody binding to optionally ligand or acceptor specifically existing in cells, tissue or organ into which the desired protein is transduced so that the desired protein of Claim 15 is transduced selectively into cells, tissue or organ.

[Claim 17]

The recombinant expression vector of Claim 15 or 16, monoclonal antibody is Fab fragment, F(ab') fragment, single strand Fv or humanized monoclonal antibody.

[Claim 18]

The recombinant expression vector of Claim 8 to 17, the vector further comprises tag sequence to facilitate purification of the fusion protein.

[Claim 19]

The recombinant expression vector of Claim 18, the vector further comprises a enzyme recognition site so that unnecessary part in biological activity regulation in cell in the desired fusion protein is cleaved.

[Claim 20]

The recombinant expression vector of Claim 19, wherein the vector comprises further six successive histidine codons.

[Claim 21]

The recombinant expression vector of Claim 20, wherein the vector comprises further the gene, which is specifically recognized and cleaved by intracellular enzyme, is Asp-Asp-Asp-Asp-Lys enterokinase cleavage site.

[Claim 22]

A fusion protein expressed in host cells comprising prokaryotic and eukaryotic cells by the recombinant expression vector of Claim 8 to 21.

[Claim 23]

A complex which the desired fusion protein of Claim 22 binds to DNA, RNA, carbohydrates, lipids or chemicals by chemical and/or physical method.

[Claim 24]

The complex of Claim 23, wherein the chemical and/or physical method is direct bonds such as covalent bond or noncovalent bond, or indirect bonds using mediates.

[Claim 25]

The fusion protein of Claim 18 or 19, wherein the desired protein is modified by ubiquitination, phosphorylation or farnesylation after translation.

[Claim 26]

A method for transducing a functional regulation material into the cytoplasm or nucleus of prokaryotes or eukaryotes comprising the follow steps:

Obtaining the complex between the peptide or its active fragment of any one of Claim 1 to 7 and biologically functional regulation material by chemical and/or physical method;

Contacting the complex to eukaryotic or prokaryotic cells in vivo or in vitro through routes such as intramuscular, intraperitoneal, intravein, oral, nasal, subcutaneous, intradermal, mucosal or inhale.

[Claim 27]

A method for transducing a functional regulation material into the cytoplasm or nucleus of prokaryotes or eukaryotes comprising the step to co-culturing the fusion protein of Claim 18 to 21 or Claim 26 or the complex of biological activity regulation material bound to the fusion protein by chemical and/or physical method with cell culture into which they are transduced.

[Claim 28]

The method of Claim 27, wherein biological activity regulation material comprises DNA, RNA, carbohydrates, lipids and chemicals.

[Claim 29]

A method for transducing biological activity regulation material into the cytoplasm or nucleus of eukaryotic or prokaryotic cell, comprising the following step:

Contacting the fusion protein of Claim 18 to 21, 26 or 28 or the complex of biological activity regulation material bound to the said fusion protein by chemical and/or physical method to cells into which the said fusion protein or the complex are transduced through routes such as intramuscular, intraperitoneal, intravein, oral, nasal, subcutaneous, intradermal, mucosal or inhale in vivo or in vitro; and

Comprising further the step adding lysosomotrophic agents to accelerate the transduction of the said material into cells.

[Claim 30]

The method of Claim 29, wherein lysosomotrophic agent is selected from the group consisting of chloroquine, monensin, amantadine and methylamine.

[Claim 31]

A recombinant expression vector having one or more DNA/RNA sequence repeatedly to which the desired DNA and/or RNA and specific proteins in its 3' terminus to be transduced into cells bind optionally using the peptide or its active fragment of Claim 1 to 7.

[Claim 32]

The recombinant expression vector of Claim 31, wherein a promoter of the expression vector is specific to cells, tissue or organ in which the desired DNA is transduced and expressed optionally.

[Claim 33]

A recombinant expression vector wherein the desired protein of Claim 8 to 17 or Claim 22 to 25 is a protein binding to DNA/RNA sequence of recombinant expression vector of Claim 31 or 32.

[Claim 34]

A fusion protein expressed in host cells comprising prokaryotic and eukaryotic cells by the recombinant expression vector of Claim 33.

[Claim 35]

The complex of Claim 34, wherein the desired protein binds to DNA, RNA, carbohydrate, lipid or chemicals by chemical and/or physical method.

[Claim 36]

The method of Claim 35, wherein the chemical and/or physical method is covalent bond or noncovalent bond, or direct bond or indirect bond using mediates.

[Claim 37]

A method for transducing the desired DNA and/or RNA into cells comprising the steps, obtaining the fusion protein and the fusion complex of DNA and/or RNA by binding the

fusion protein of Claim 34 or 35 to the desired DNA of Claim 31 or 32; and co-culturing and contacting the said complex with cells, into which the desired DNA and/or RNA is transduced.

[Claim 38]

A method for transducing the fusion complex of the desired DNA of Claim 31 or 32 and the fusion protein of Claim 34 and 35 into the cytoplasm or nucleus of eukaryotic or prokaryotic cells in vivo through routes comprising intramuscular, intraperitoneal, intravein, oral, nasal, subcutaneous, intradermal, mucosal or inhale.

[Claim 39]

A recombinant expression vector, to which an expression is bound operatively, wherein the vector has ordely the DNA coding transducible peptide or its active fragment and the desired protein of Claim 34 and 35, the amino acid sequence to be cleaved by protease existing on surface of cells(for example, MMP(MatrixMetalloProtease etc.) and the DNA coding ligands or receptors specifically existing on cells, tissue or organs into which the desired DNA/RNA is tranduced optionally, in order to transducer the desired DNA of Claim 31 or 32 and the fusion protein of Claim 34 and 35 or the fusion complex into cells, tissue or organs optionally.

[Claim 40]

A recombinant expression vector coding monoclonal antibody which optionally binds to ligands or receptors specifically exisiting on cells, tissue or organs into which the DNA coding the transducible peptide or its active peptide of Claim 34 and 35 and the desired protein, the amino acid sequence specifically to be cleaved by protease existing on surface

of cells (for example, MMP(MatrixMetalloProtease etc.) and the desired DNA/RNA are transduced.

[Claim 41]

A complex prepared by binding through chemical covalent or noncovalent or physical method with monoclonal antibody which optionally binds to ligands or receptors specifically existing on cells, tissue or organs into which the DNA coding the transducible peptide or its active fragment and the desired protein, the amino acid sequence to be specifically cleaved by protease on surface of cells (for example, MMP(MatrixMetalloProtease etc.) and the desired DNA/RNA are transduced optionally in order to transduce the desired DNA of Claim 31 or 34 and the fusion protein of Claim 34 and 35 or the fusion complex optionally.

[Claim 42]

A recombinant expression vector, wherein monoclonal antibody of Claim 40 or 41 encode Fab fragment, F(ab') fragment, single strand Fv or humanized monoclonal antibody.

[Claim 43]

A fusion protein expressed in a host cell comprising prokaryotic and eukaryotic cell by recombinant expression vector of Claim 39 to 42.

[Claim 44]

A recombinant expression vector further comprising spacer amino acid (one or more glycine, amino acid AAY etc.) or nucleic acid for flexibility of the protein encoded in each gene or for stability of entire construct and function of the fusion protein between genes

prepared operatively from the expression regulation sequence in the recombinant expression vector of Claim 8 to 21, 31 to 33, and 39 to 42.

[Claim 45]

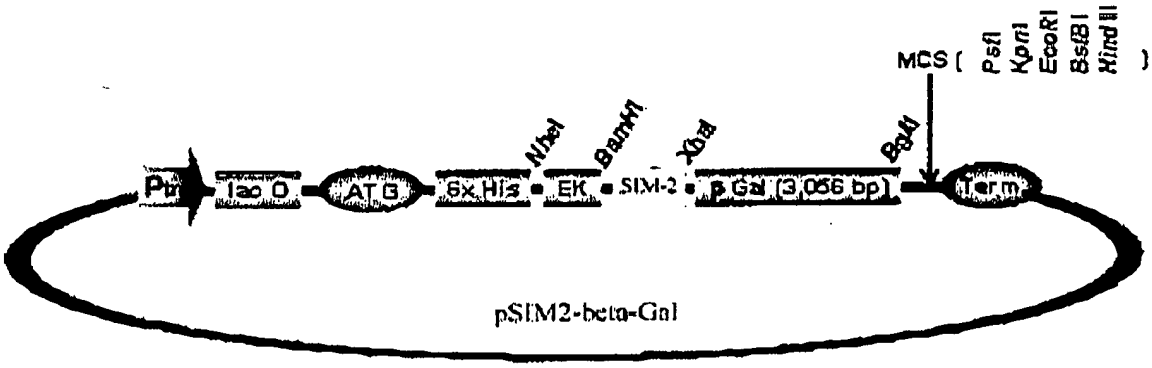
A fusion protein expressed in the host cell comprising prokaryotic or eukaryotic cells by the recombinant expression vector of Claim 44.

[Claim 46]

A method for transducing molecules into cells, characterized by co-delivery of biologically active molecules such as cytokine (IL-4, IL-12 or gamma-IFN etc.) or growth factor (EGF, IL-2 etc.) which can regulate cellular physiological phenomenon more effectively by the transduction into cells, when the desired fusion protein or the desired DNA/RNA is transduced into cells in vivo or in vitro by the peptide or its active fragment of any one of Claim 1 to 7, which is a transducible peptide of Claim 7, 13, 26, 27, 29, 37 and 38.

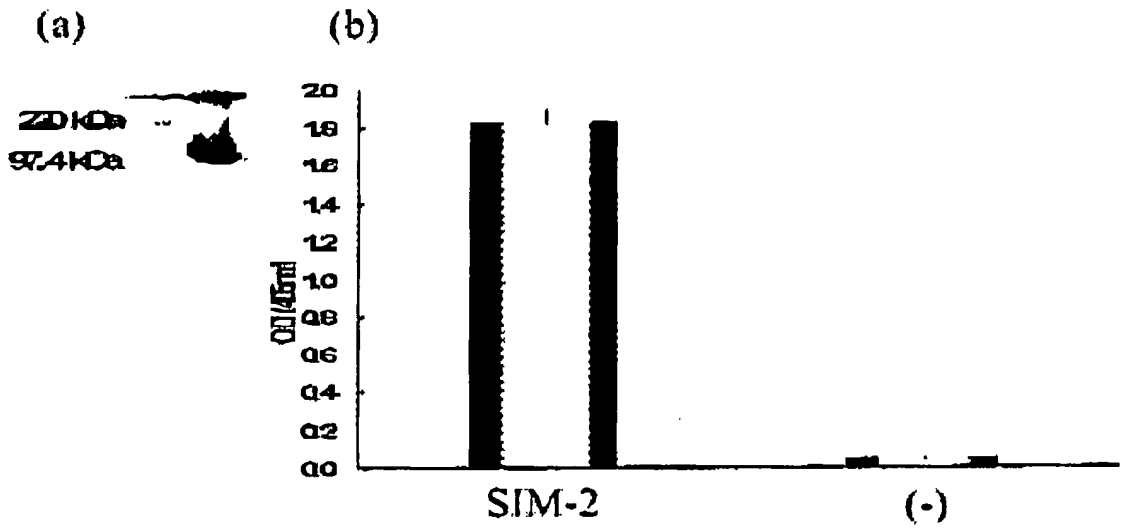
[DRAWINGS]

[Figure 1a]

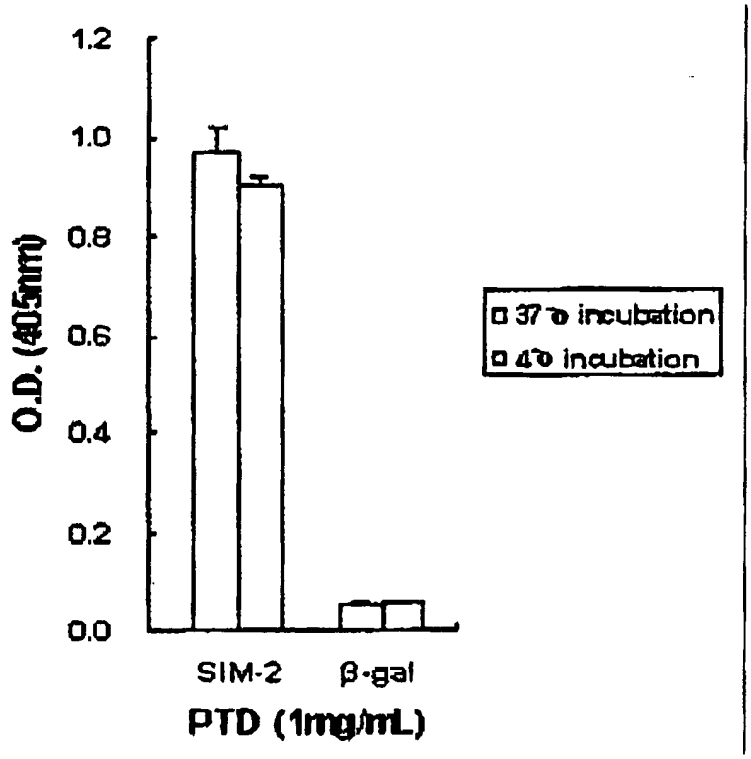


[Figure 1b]

[Figure 2]



[Figure 3]



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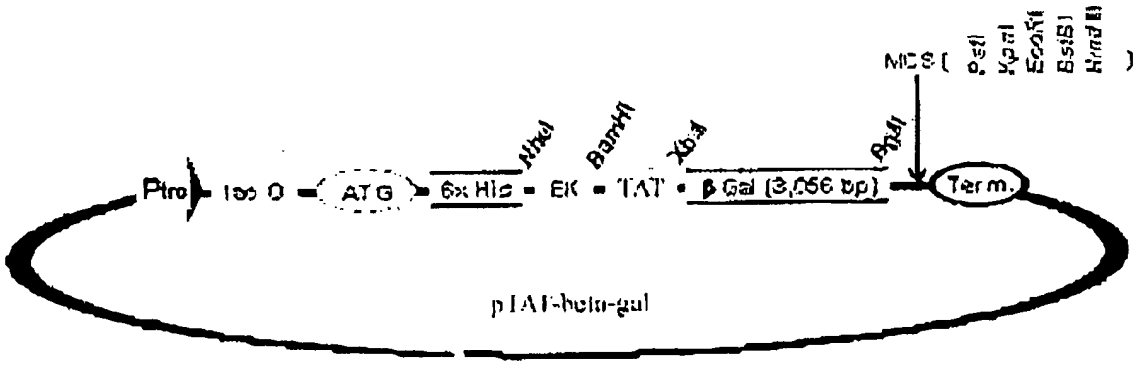
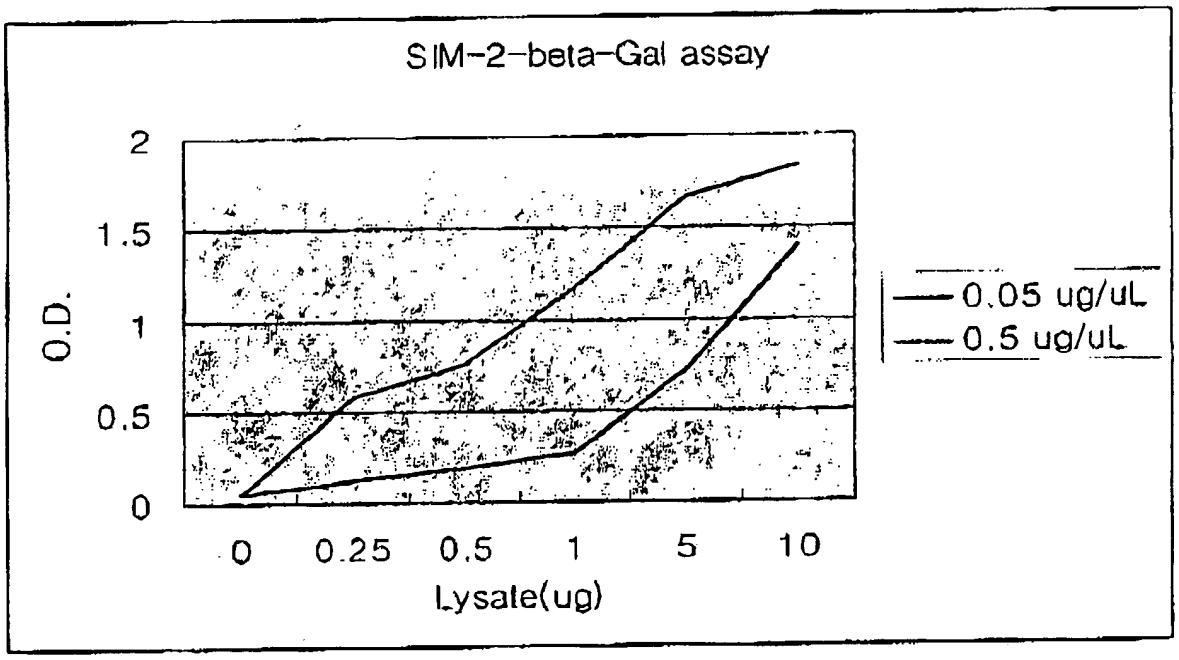
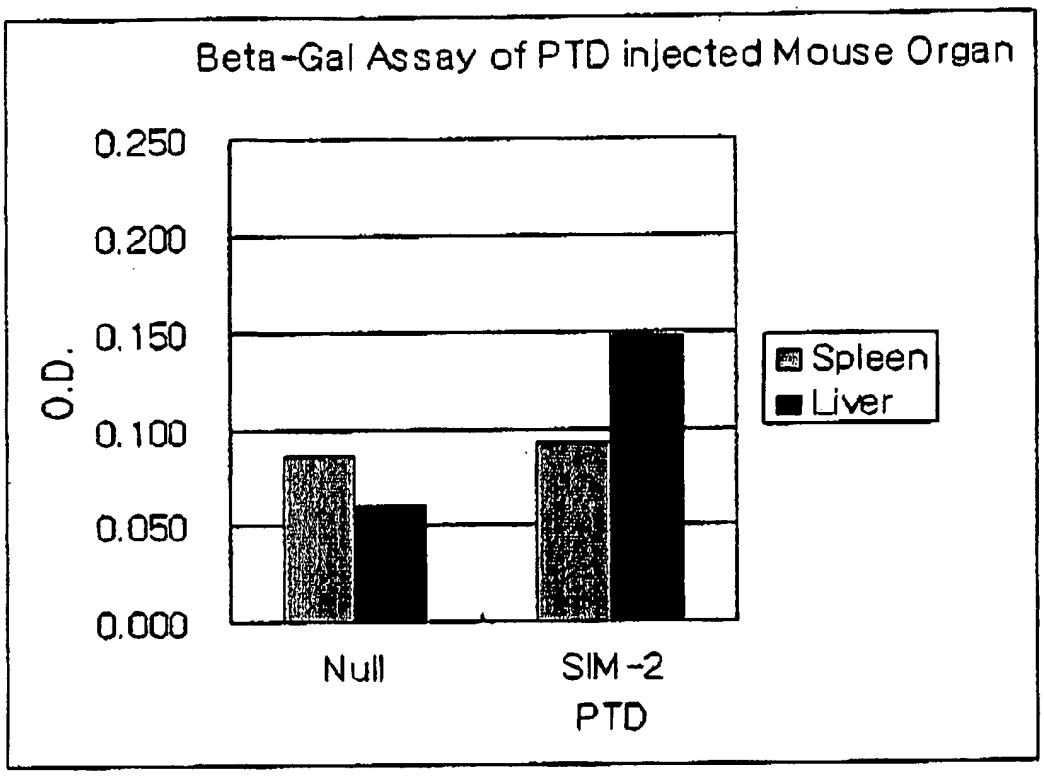
[Figure 4a]



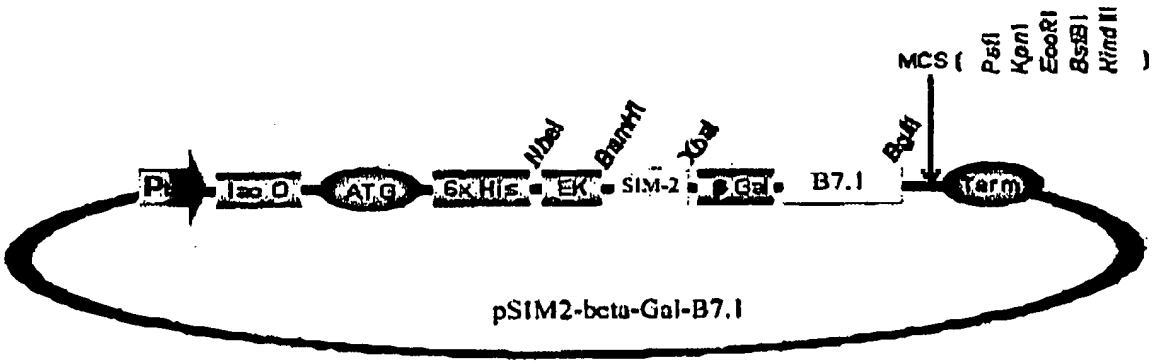
[Figure 4b]



[Figure 5]



[Figure 6a]



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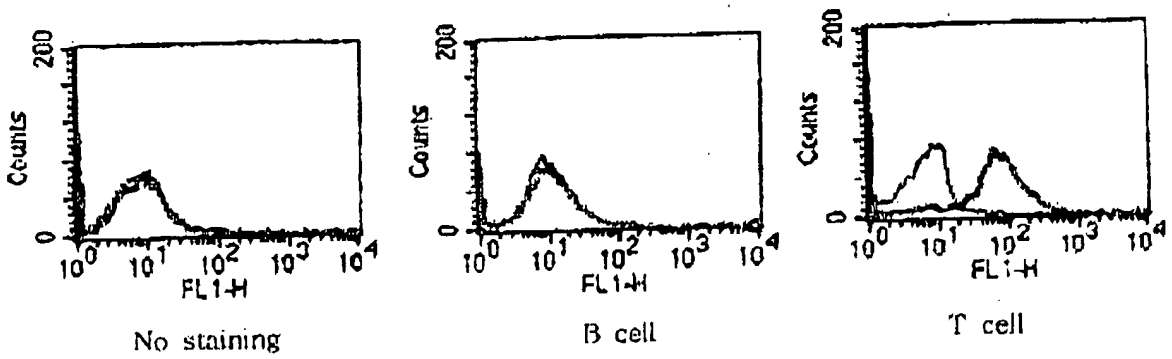
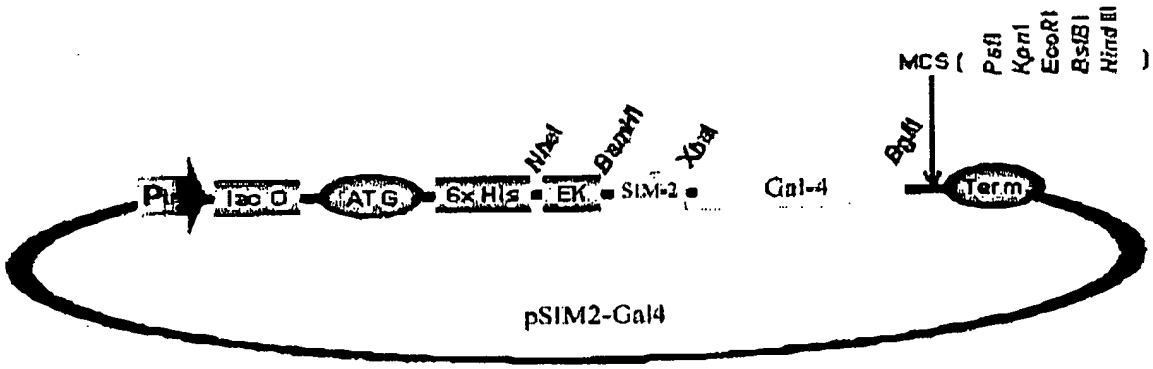
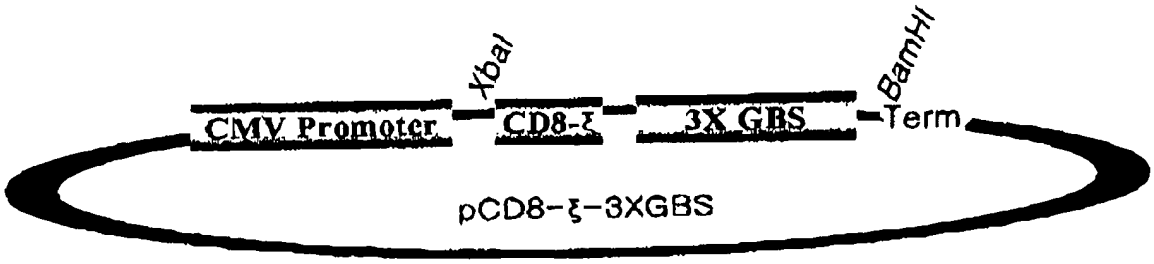
[Figure 6b]



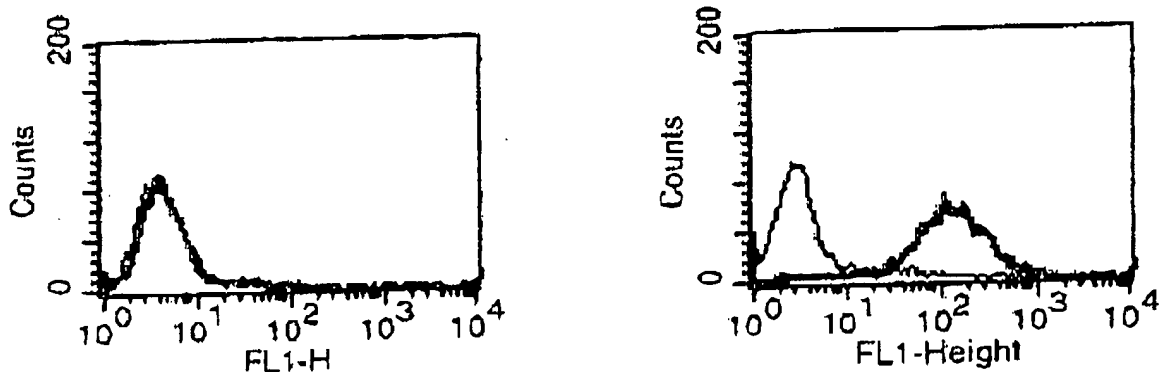
[Figure 7]



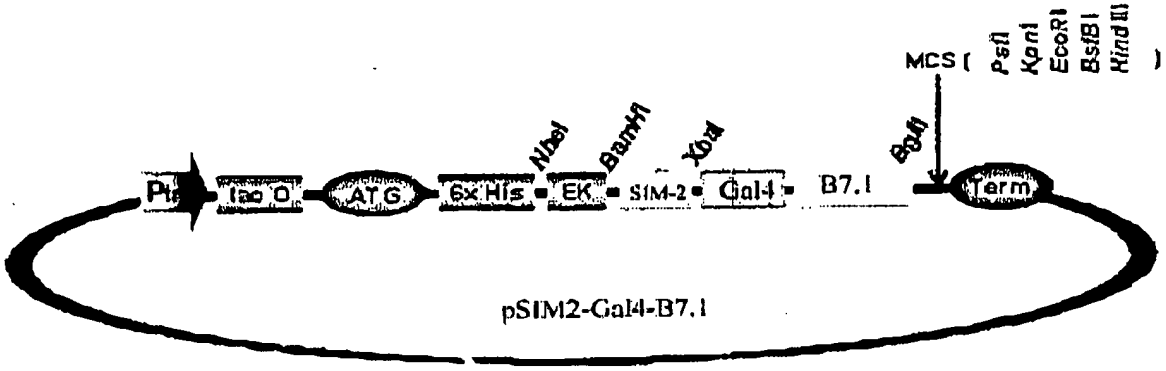
[Figure 8]



[Figure 9]



[Figure 10a]



[Figure 10b]